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CHAPTER 45

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Phenylacetic Acid Metabolism by Three Aquatic Bacteria Isolated from Continuous Culture Enrichments

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The fate of toxic organic compounds in aquatic environments depends, in part, on the capacity of microorganisms to metabolize these compounds at very low concn. Little information exists on the fate of aromatic ring compounds at low concn (< 100 mg/l) in aquatic environments. Therefore, we attempt to characterize the degradation of the aromatic compound phenylacetic acid (PAA) at low concn by aquatic bacteria. Three bacterial isolates, PAL-1, PAL-10, and PAL-100, obtained from continuous culture enrichment experiments at PAA concn of 1, 10, and 100 mg/liter, respectively. Washed cell suspensions of each isolate metabolized PAA, as measured by oxygen uptake, without lag regardless of whether cells were grown in acetate or PAA minimal salts medium, suggesting PAA metabolism was constitutive. Acetate-grown PAL-1 and PAL-100 cultures showed greater oxygen uptake activity in the presence of PAA than did PAA-grown cultures. All three isolates had high activity in the presence of meta-hydroxy-PAA. Incubation of washed cell suspensions at 25 C for 12 h showed that only the PAL-1 isolate lost activity (75%) relative to cell suspensions incubated at 4 C. Our studies provide physiological criteria that may characterize the types of bacteria that transform aromatic compounds at low concn in aquatic environments.

INTRODUCTION

paucity of information exists on microbial degradation of aromatic compounds at low ncn (0.1-50 mg C/1) in aquatic environments (Floodgate 1976). Extensive concern over the te of toxic aromatic compounds (i.e., chlorinated hydrocarbons, polynuclear aromatic ydrocarbons, pesticides) in aquatic ecosystems demands a more complete knowledge of etabolic potentials in indigenous aquatic bacterial populations. Laboratory experiments esigned to study the metabolic potential of aquatic bacteria often employ relatively high vels of aromatic substrates (1000 mg C/1). Since many aquatic environments typically ave low concn of these organic aromatic materials (Croll 1972), the information generated om these investigations is difficult to extrapolate to natural situations. Jannasch (1968, 967) studied the transformation of simple organic substrates (lactate, glycerol) and showed rough the use of continuous culture enrichment that many aquatic bacteria are physio- gically adapted for efficient growth at low organic substrate concn. Similar studies, how- er, have not been reported for aromatic compounds.

This paper reports on a preliminary study of the physiology of bacteria obtained by con- nuous culture enrichments on low concn of the aromatic compound phenylacetic acid PAA). PAA was chosen as the model substrate because its bacterial metabolism by

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laboratory cultures has been studied (Sparnins et al. 1974; Blakley et al. 1967); it is also represenative of the types of aromatic compounds used for manufacture of certain herbicides and toxic organics (Sheets et al. 1968).

Materials and Methods

Enrichments. Continuous culture enrichment experiments followed the procedure of Jannasch (1967), and used a single-stage, 1-liter growth vessel with a 600-ml working volume. Volume was maintained by a positive air pressure overflow and contents of the growth vessel were mixed with a magnetic stirrer. Reservoir medium was peristaltically pumped (Model 1203 pump, Harvard Apparaturs Co.,[1] Millis, MA) into the continuous culture system through a vapor barrier to prevent back-contamination of reservoir fluid.

Carbon and nutrient supplements for reservoir media were sterilized separately and added aseptically to sterile glass carboys containing 10 liters of sterile deionized water. Reservoirs were prepared to yield influent PAA concn of 1, 10, or 100 mg/liter. Nutrient supplements, NH_4 NO_3, $MgSO_4 \cdot H_2O$ potassium phosphate buffer (pH = 7.3), were added to the reservior medium at a C:N:P:Mg ration of 10:4:1:1. Based on C:N:P:Mg ratios in Lake Ontario (Rae and Dutka 1974), this assured carbon was the only growth-limiting nutrient.

Enrichment experiments were initiated by placing a 600-ml sample of fresh Lake Ontario water (collected with sterile bottles along the shoreline of Lake Ontario State Park) in a continuous culture growth vessel and supplementing with PAA, phosphate buffer (pH 7.3), ammonium nitrate, and magnesium sulfate at 10 times the reservoir concn (this initiated growth of all bacteria capable of growth on PAA and ultimately shortened the length of the enrichment experiment). The vessel was then incubated as a batch culture, with stirring and aeration (filter-sterilized air) for 12 h. Thereafter, flow of reservoir medium was commenced at a dilution rate (D) = $0.1 h^{-1}$. All systems were incubated at room temp, 25 ± 2 C.

Changes in population composition during the experiments were monitored by standard plate count techniques. One-milliliter samples (1 ml) were periodically removed aseptically from the growth vessels, diluted serially in sterile dilution blanks (deionized water), and plated, by spreading, on PAA minimal salts (PMS) agar medium that contained 1.0 g PAA, 0.8 g NH_4NO_3, 0.07 g $MgSO_4 \cdot H_2O$, 0.03 M potassium phosphate buffer (pH = 7.3), and 15 g Bacto agar per liter of deionized water. The outcome of an enrichment experiment was determined by examining the relative proportion of colony morphological types.

After 10 turnover volumes, the predominant colonial morphological type from each enrichment experiment (i.e., PAA reservoir concn of 100, 10, or 1.0 gg/1) was streaked for purity on PMS agar and stored at 10 C on PMS agar slants.Slant cultures were transferred every 4 mo in order to maintain original physiological characteristics. Bacteria representing each colony type were characterized by tests cited in Buchanan and Gibbons (1974).

Oxygen uptake studies. Oxygen uptake was measured with a Gilson Oxygraph (Gilson Medical Electronic, Inc., Middleton, WI) equipped with a Clarke-type electrode. Cells to be studied were grown in batch culture on either PMS broth or minimal salts broth (see above) containing 0.1% sodium acetate.When all cultures had reached stationary phase (about 30

[1]Mention of trade names or commercial products does not constitute endorsement by EPA.

h), cells were harvested by centrifugation at 12,100 × *g*, washed twice with, and resuspended in, a 0.05 M phosphate buffer (pH 7.3). Protein content of all cell suspensions was determined by themethod of Lowry et al. (1951) using bovine serum albumin as the standard. Oxygen uptake was measured on cell suspensions containing 50–100 μg of protein. Substrates for oxygen uptake studies were prepared in deionized water (adjusted to pH 7.3) and added to cell suspension at final concn of 3.8 mM PAA, 3.3 mM *p* -hydroxy-PAA (*p*-OH-PAA) and 1.6 mM *m*-hydroxy-PAA). Uptake rates were corrected for endogenous activity and expressed in specific activity units of nM O_2/min/μg protein.

All chemicals used in this study were reagent grade. Phenylacetic acid was purchased from Eastman Organic Chemicals (Rochester, NY) and *m*-hydroxy and *p*-hydroxy phenylacetic acids were purchased from Aldrich Chemical (Milwaukee, WI).

RESULTS

Enrichments. Within 10 turnover volumes (100 h), continuous culture enrichment experiments at PAA reservoir concn of 1, 10, or 100 mg/liter showed a dominant (50–60% of total population) colony type at each concn. The rapid appearance of a predominant colony morphological type suggested that PAA was metabolized readily by aquatic bacteria. Table 1 shows a partial taxonomic characterization of the bacteria representative of each predominant colony type. The bacterial isolates (hereafter referred to as PAL-1, PAL-10, or PAL-100) appeared to be members of the genus *Pseudomonas.* The all grew in minimal salts broth containing PAA, *p*-OH-PAA, *m*-OH-PAA, or benzoate (very slow growth).Each was readily distinguishable on the basis of growth in nutrient broth or colony morphology.

TABLE 1. *Partial characterization of isolates from continuous culture enrichment experiments*

Test	Isolate PAL-1	PAL-10	PAL-100
Morphology	Gram-neg. rod small, motile	Gram-neg. rod small, motile	Gram-neg. rod large, motile
Growth at 4 C	-	-	-
Growth in nutrient broth	poor	+	+
Ocid/ferm test	strict aerobe	strict aerobe	facultative
Starch hydrolysis	-	-	-
Gelatin hydrolysis	-	+	-
NO_3 reduction	++	-	-
Pigments	-	green on aromatic acids	-
Growth on acetate	+	+	+
Growth on succinate	+	+	+
Poly-β-hydroxy-butyrate accum.	-	-	-
Oxidase	+	+	-

Oxygen uptake studies. Rates of oxygen uptake for washed cell suspensions harvested from cultures with either sodium acetate or PAA as the sole source of carbon and energy are shown in Table 2. Variation in uptake rates was never more than ± 5% for any single washed cell suspension regardless of the substrate tested. Washed cell suspensions maintained at 4 C gave good reproducibility (± 5%) of uptake rates even with 48-h-old suspensions. Absolute rates of oxygen uptake by washed cell suspensions of the same organism prepared on different days were variable and comparisons could not be made. Variation depended largely on the length of the incubation period before harvesting the cultures. Rates were determined from the slope of the uptake curves. These curves were lniear for at least 15 min after addition of substrate to washed cell suspensions. A fivefold increase in substrate concn had no effect on oxygen uptake, indicating substrate was in excess and maximal uptake rates wree being observed.

Oxygen uptake by washed acetate-grown cells in the presence of PAA, *p*-OH-PAA, or *m*-OH-PAA (Table 2) occurred without lag. The addition of choloramphenicol (50 mg/ml), a protein synthesis inhibitor, had no effect on oxygen uptake regardless of when it was added to washed cell suspensions, which indicated the uptake was not due to the synthesis of new enzymes. Our results suggest that PAA oxidation in these aquatic isolates is a constitutive process, i.e., enzymes for PAA metabolism are present in cells grown in the absence of PAA. Since specific enzymological studies were not carried out, it is possible that oxygen uptake by acetate-grown cells when given PAA was due to the metabolism of a chemical contaminant (such as acetate in the PAA stock solution). However, the use of purified PAA stock solutions (99%) did not alter the constitutive oxygen uptake response.

In several cases, specific activities of acetate-grown cells were higher than for PAA-grown cells (Table 2). This phenomenon was most pronounced with the PAL-1 isolate where all

TABLE 2. *Rates of oxygen uptake by washed cell suspensions on phenylacetic acid*

Isolate[a]	Substrate[c]	Specific Activity[b]	
		Acetate Grown Cells[d]	PAA Grown Cells[d]
PAL-1	PAA	0.169	0.051
	p-OH-PAA	0.084	0.025
	m-OH-PAA	0.169	0.096
PAL-10	PAA	0.094	0.363
	p-OH-PAA	0.044	0.056
	m-OH-PAA	0.135	0.146
PAL-100	PAA	0.169	0.270
	p-OH-PAA	0.127	0.041
	m-OH-PAA	0.127	0.102

[a]Numbers (1, 10, 100) refer to reservoir concn (mg/*l*) of PAA during continuous culture enrichment experiments from which these isolates were obtained.

[b]nM O_2/nub/„g protein.

[c]PAA = phenylacetic acid, *p*-OH-PAA = parahydroxy phenylacetic acid, *m*-OH-PAA = metahydroxy phenylacetic acid.

[d]Cells were harvested from cultures grown in minimal salts broth containing either PAA or sodium acetate as the sole source of carbon and energy.

three substrates were more rapidly oxidized by acetate-grown calls. PAL-100 displayed similar activities but only with *pOH-PAA and m*-OH-PAA. PAL-10 cells grown on PAA always showed higher specific activities on the three substraters.

The rapid and immediate uptake of oxygen by all three isolates (PAA or acetate-grown cells) in the presence of *m*-OH-PAA (Table 2) was unexpected. Previous studies on PAA metabolism by *Pseudomonas* (Blakley et al. 1967; Kunita 1955) indicated that cells grown on PAA are not simultaneously adapted to the utilization of *m*-OH-PAA. In our studies *m*-OH-PAA was metabolized equally as well as *p*-OH-PAA.

Several times in our studies, washed cell suspensions of the PAL-1 isolate exhibited substantial reductions in oxygen uptake activity. To verify this instability, washed cell suspensions of all three isolates were monitored for changes in specific activity following a 12 h incubation at 25 C in phosphate buffer (pH 7.3). Control cell suspensions were simultaneously maintained at 4 C (ice bath) for the same period. PAL-1 lost 75% of the original oxygen uptake activity (Table 3) on both PAA and *m*-OH-PAA compared to the control which was not significantly different from the preincubation activity. However, PAL-10 and PAL-100 showed no loss in activity after incubation at 25 C. Acetate- and PAA-grown cells were affected in the same manner. Other characteristics such as small cell size and poor growth in PAA minimal salts broth or nutrient broth (Difco) contrasted the PAL-1 isolate with the other two.

Discussion

Manometric studies on whole cells, although not as definitive as enzymological studies, provide a simple means of distinguishing physiological differences between aquatic bacertia growing on phenylacetic acid. Our results demonstrate that bacteria isolated during these continuous culture enrichment studies have two unusual qualities: (1) they appear to be constitutive for PAA oxidation (i.e., acetate-grown cells take up oxygen without lag in the presence of PAA); and (2) the rates of PAA oxidation (i.e., rates of oxygen uptake) were faster on acetate-grown cells than on PAA-grown cells. We suggest these qualities are in-

TABLE 3. *Effect of 12 h incubation at 25 C on oxygen uptake activity of washed cell suspensions[a] acetate-grown cultures*

Condition	Substrate	Specific Activity[b] PAL-1	PAL-10	PAL-100
Preincubation - 4 C	PAA	0.170	0.038	0.168
Post incubation - 4 C		0.169	0.038	0.169
" " - 25 C		0.043 (25%)[c]	0.037	0.169
Preincubation - 4 C	*m*-OH-PAA	0.376	0.176	0.130
Post incubation - 4 C		0.376	0.178	0.127
" " - 25 C		0.096 (25%)[c]	0.179	0.127

[a]Incubation in 0.05 M phosphate buffer (pH 7.3) at 25 C for 12 h.
[b]nM O_2/min/μg protein.
[c]Percentage of original activity.

dicative of metabolic processes that typify aquatic bacteria capable of efficient growth at low organic substrate concn.

Two types of initial hydroxylation reactions are employed by microorganisms for the aerobic degradation of PAA; one results in the production of the intermediate homogentistic acid (Kunita 1955), whereas the second generates homoprotocatechuic acid (Blakley et al. 1967). The aromatic ring of both intermediates is then cleaved by specific dioxygenases typical of the *meta* fission pathway. Since our isolates oxidized PAA better when grown in acetate minimal salts medium, it could be reasoned that the acetate functional group of PAA was being metabolized before hydroxylation of the ring. However, initial analysis of metabolic end products employing ^{14}C-carboxyl-labeled PAA (Mahaffey, unpubl. data) has provided no evidence for this pathway.

Hegeman (1966) showed that mutants of *Pseudomonas putida*, constitutive for synthesis of mandelate dehydrogenase, became superinduced (i.e., produced enzyme concn greater than those observed in induced wild-type cells) when their carbon and energy source was exhausted. With theisolates used in our study, sufficient cell densities for oxygen uptake measurements could be obtained only by harvesting cells from stationary phase cultures; i.e., when PAA was completely consumed. This condition is similar to that described by Hegeman (1966) and may account for the superinduced oxygen uptake activities that we observed.

It is possible tht the constitutive PAA metabolism evident in our acetate-grown cells could have resulted from selection of a mutant during continuous culture enrichment (Horutchi et al. 1962). However, mutant selection in a continuous culture system, where substrate concn and dilution rates are kept low, requires a minimum of 30-40 turnover volumes. The aquatic bacteria employed in our studies were selected during the first 10 turnover volumes and thus, we feel they were genetically unaltered during the enrichment experiment and were representative of the field population.

Lucyszyn and Pritchard (*Developments in Industrial Microbiology*, this volume) studied a *Pseudomonas* sp. isolated by continuous culture enrichment at low lactose concn (5 mg/1) which was unable to maintain viability (decrease in colony-forming units) when washed cells were suspended in phosphate buffer at 25 C. Similar effects (as measured by loss of O_2 uptake activity with PAA) were also noted in our study of phenylacetic acid-degrading isolates. The effect, however, was confined to the PAL-1 isolate (obtained from an enrichment experiment at 1 mg/1 PAA) and perhaps further distinguishes a physiological property typical of aquatic bacteria that grow at low carbon source concn. We have no explanation for this relative instability but its elucidation will lead to a better understanding of indigenous aquatic microorganism and the transformation processes they mediate.

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